



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : G01N 15/00, B04B 5/04, 7/02, G01N 33/48	A1	(11) International Publication Number: WO 99/23471 (43) International Publication Date: 14 May 1999 (14.05.99)
<p>(21) International Application Number: PCT/EP98/06865</p> <p>(22) International Filing Date: 29 October 1998 (29.10.98)</p> <p>(30) Priority Data: MI97A002457 31 October 1997 (31.10.97) IT</p> <p>(71)(72) Applicant and Inventor: SITAR, Giammaria [IT/IT]; Corso Partigiani, 32, I-27012 Certosa di Pavia (IT).</p> <p>(74) Agent: GERVASI, Gemma; Notarbartolo & Gervasi S.p.A., Corso di Porta Vittoria, 9, I-20122 Milan (IT).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: DEVICE AND METHOD FOR THE SEPARATION OF HUMAN OR ANIMAL CELLS OF DIFFERENT DENSITIES FROM CELLULAR DISPERSIONS WHICH CONTAIN THEM</p> <p>(57) Abstract</p> <p>The invention concerns a device and a method which allow the efficacious and rapid separation of organic cellular populations of different densities, from the dispersions which contain them:</p>		

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DEVICE AND METHOD FOR THE SEPARATION OF HUMAN OR ANIMAL CELLS OF DIFFERENT DENSITIES FROM CELLULAR DISPERSIONS WHICH CONTAIN THEM

Summary

- 5 The invention concerns a device and a method which allow the efficacious and rapid separation of organic cellular populations of different densities, from the dispersions which contain them, said device comprising an elongated chamber whose cross section decreases from the base towards the top of the device and said method comprising the introduction of a dispersion of a cell population into
10 said chamber of the device previously filled with a liquid whose density varies from a maximum, near the device base, to a minimum near the top, subjecting the device with the cellular dispersion to centrifugation and collecting the separated fractions by introducing a very dense water immiscible liquid at the base of the separator device while simultaneously pumping a gas at the same pressure as
15 said very dense liquid, to the device top so that the layered cellular fractions inside the chamber are expelled through at least one hole situated at an intermediate level of said chamber length.

Prior art

The invention refers to both a device and method for the separation of human or
20 animal cellular fractions of different densities, from cell populations present in cellular dispersions.

It is known that human or animal cells have differing densities (generally between 1.04 g/cm³ and 1.1 g/cm³) and that each cell type has its own characteristic density.

25 It is also known that it is often important and necessary to separate differing cell types for possible concentration or purification of the same, from a dispersion of a heterogeneous population of cells. This is fundamental in making the biochemical and functional analyses possible on both normal cells and neoplastic populations.

The finer and more careful the separation of different density cells, and the more rapid is the separation operation then the data obtained will be equally good and useful, with consequent benefits to the practical procedure.

If one considers, for example, that certain types of cells are present in the blood or human tissues in extremely reduced quantities, it is evident that from their effective and rapid identification, the possibility of chemical, biochemical or medical intervention, otherwise impossible or only hypothetical, could follow.

The most advanced cell separation technique in current use employs a separator device consisting of a casing with an internal cavity. The latter is laterally delimited by tapered surfaces, with the greatest cross section near the end or base from which a tube or duct end is connectable to a peristaltic pump. The cavity is tapered towards the opposite end or top of the casing, which has an outlet hole. Initially, a liquid is introduced into the base cavity of the separator device using the peristaltic pump and a gradient mixer. The liquid density slowly increases as the quantity of liquid in the cavity increases, until filling it. Thus, the separator device cavity is filled with a liquid which is practically made up of successive and continuous strata of liquid, increasing in density from the top to the base of the device cavity. At this point, the liquid supply is suspended and a dispersion of the cellular population to be separated into homogeneous cellular fractions by density is introduced through the hole in the top, until the upper cavity section is filled: at the same time as the dispersion introduction, a corresponding volume of the liquid first introduced is drawn off from the separator device base, so that the cellular population dispersion can fall into the device cavity.

The separator device is then closed in a centrifuge, with the rotation axis perpendicular to the separator device axis and near its top, during centrifugation. In the centrifuge, a very large force is transmitted to the liquid closed in the device (for example 400g), resulting in the distribution of the cellular population dispersion in the liquid in the form of cellular bands (or strata) of differing densities.

- The separator device is then removed from the centrifuge and a very dense water immiscible liquid is introduced into the base causing the expulsion (from the hole in the top of the same device) of the fractionated cellular dispersion as described: the desired number (for example 10 or 12) of distinct dispersion fractions with an increasing density gradient are extracted through the separator device top, the cells present in each fraction having different densities to the adjacent fractions .
- 5 The device and the methodology briefly outlined above, are described in detail by Giannmaria Sitar and Piermaria Fornasari in HAEMATOLOGICA, Vol. 74, N1, Jan-Feb. 1989; and by Pretlow II TG and Pretlow TP eds. Cell Separation: method and
- 10 selected applications, New York, Academic Press, 1982 (5 volumes).
- The recovery system of the different separated cell fractions described above, has its limits, however, and presents some serious problems. Infact, the collecting of the different and subsequent cell fractions is carried out through a small hole in the separator device top, under the push of a dense liquid which is pumped in at the device base. This causes a hydrodynamic disturbance in the different adjoining strata where the cell density is different, and causes the widening (well known to field technicians) of the strata or bands where the different density cells are collected or distributed, resulting in the contraction of the liquid strata separating the bands where are collected the cells of mainly homogeneous
- 15 density and different from band to band, which can even lead to the remixing of the various cellular fractions that were separated during centrifugation. This phenomenon is more serious and unacceptable, the narrower the bands, in which the cells have been collected, and the smaller the distance separating one cell from another.
- 20
- 25 In order to try and minimise these problems, the pressurised liquid at the base of the separator device is introduced with extreme slowness, a fact which leads, however, to long and unacceptable times necessary for the collection of the different cellular fractions. See the publications of Giannmaria Sitar and Piermaria Fornasari already mentioned; and TULP, Anal. Biochem. 1981, vol. 117, pages
- 30 354-365.

Brief description of the drawings

- Figure 1 is a schematic view of a known separator device of cells;
- Figure 2, represents, a schematic view of a separator device according to the invention of which:
- 5 - Figures 3 and 4 are longitudinal sections of the device according to the lines 3-3 and respectively 4-4 of Figure 2.

Description of the invention

The main aim of the present invention is that of finding a device and method which allow the easy and very clear collection of cellular fractions which have been separated in the device after centrifugation.

Another aim is that of allowing the recovery of the different cellular fractions in shorter times than is currently possible, making the collection of useful data possible, which would otherwise have proved useless.

The above aims, along with others, are satisfied with a separator device with a base and top, containing an elongated chamber, whose cross section decreases from the base towards the device top, the latter containing at least a first channel, one end of which opens onto the inside of the said chamber near the said base, the other end connectable to a pressurised liquid source and a second channel, one end of which opens into the same chamber corresponding to the device top, characterised by the fact that in the said device there is at least an additional channel, one end of which opens along the said chamber length, the other end opening onto the exterior of the device.

The invention also concerns the separation method of cellular fractions from dispersions of human or animal cell populations, in which, first of all, the dispersion of a cell population is introduced into the separator device chamber, previously filled with a liquid whose density varies from a maximum near the device base and a minimum near the top. The device is then subjected to centrifugation so as to distribute the cells into distinct fractions with differing densities, the method being characterised by the fact that the separated fraction recovery occurs by introducing, in the device base, a very dense, water immiscible liquid while simultaneously pumping a gas to the top (at the same delivery

pressure as the very dense liquid), so that the layered cellular fractions inside the chamber are expelled through at least one hole situated in an intermediary zone along the said chamber length.

It was found, that proceeding in the above manner the cell bands, which were
5 found layered in the separator device, were compressed after centrifugation
(instead of expanding, as happens with the known method) in the same chamber,
in a way that the various bands could be recovered separately in a clear manner,
being expelled from the chamber (through the holes located laterally to the same
chamber) without undergoing any hydrodynamic disturbance, i.e. there is no cell
10 band remixing with those immediately adjacent.

It was also ascertained that, by proceeding in the manner described, the various
cellular fractions could be separated and recovered at greater speeds than with
the known technique, and cellular fractions are not mixed, as already mentioned.
In order to make clearer both the device structure and the invention procedure,
15 there will now follow the description of a potential application, as an unlimiting
example, with reference to the enclosed figures.

Reference is made, first of all, to Figure 1 which illustrates a separator device of
known structure made of an elongated casing 1 with an internal bell cavity 2
delimited by a substantially conical surface (dotted lines) with a greater cross
20 section towards its base (with respect to the diagram) where there is the base 3
body affixed, which seal closes the cavity 2. Casing 1 is crossed by a duct 4 from
whose upper end, a tube 5 extends, whose lower end opens inside cavity 2
through the channels 6 located in base 3 casing which is fixed to casing 1 in a
suitable way, for example, with a series of bolts (not shown) or screw connection.
25 According to the present invention near the base of the device a flow deflector 11
is provided to disperse evenly the incoming fluid arriving from the channels 6,
through the entire cross section of the inside chamber 2.

Cavity 2 is tapered towards the top of casing 1 which is crossed by a duct 7 linking
the cavity with tube 8.

When one wishes to use the illustrated device, cavity 2 is filled, in the known way using tube 5, duct 4 and channels 6, with a liquid whose density gradually increases as it is introduced into the cavity, until filling it.

Thus cavity 2 is filled with a liquid which is practically made up of successive and 5 continuous strata of liquid with increasing densities from the top to the base of the device. The liquid supply, through tube 5, is then stopped and the cellular population dispersion to be separated into homogeneous cellular fractions by density is introduced in cavity 2, while a corresponding volume of liquid is drawn off through tube 5.

10 The separator device is then placed in a centrifuge at high speed until the distribution of the cellular population dispersion in the liquid, in form of cellular strata of different densities, is obtained.

At this point, a very dense water immiscible liquid is introduced into cavity 2, through tube 5, causing the expulsion of the cellular dispersion fractionated in the 15 manner described from tube 8 collecting the desired number of distinct cellular dispersion fractions of increasing density gradient.

The recovery system of the different cell fractions described above with reference to Figure 1, presents the serious problem that the expulsion of the differing cellular fractions by tube 8 occurs entirely with the push of the dense liquid coming out 20 from channels 6 at the device base, with the consequent well known contraction or thinning of the liquid strata separating the bands where are collected the cells of homogeneous density but different from band to band, (resulting in difficulties of separate collection of the different bands). This can lead to serious and unacceptable remixing of the differing cellular fractions that were separated during 25 centrifugation.

To minimise the above problem, the introduction of the liquid at the base of cavity 2 is carried out with extreme slowness, which means long times are necessary for the recovery of the different cellular fractions which is extremely serious as known to the field technicians.

30 Figures 2 to 4 illustrate a separator device according to the present invention.

This separator device is similar to that in Figure 1: for clarity, the same reference numbers used in Figure 1 have been adopted for Figures 2 to 4 to illustrate the various parts.

The separator device of Figures 2 to 4 is different from that of Figure 1 for the fact
5 that two supplementary ducts 9, are located in casing 1 (in reality, there could be more than two, or even one) from which a tube 10 extends from the upper end, whose lower end opens into the inside of cavity 2 halfway along its length.

The filling and centrifugation procedures of the device in Figure 2 to 4 have already been described with reference to Figure 1 and will not therefore be
10 repeated here. The recovery stage of the different separated cellular fractions from the device is very different.

In fact, the collection of the separated fractions occurs by introducing a very dense water immiscible liquid at the base of cavity 2 (as in the known technique) while simultaneously pumping a gas (for example, air) through tube 8 to the top at the
15 same pressure as the dense liquid, so that the layered cellular fractions inside cavity 2 are compressed and forced out through ducts 9.

It was found, proceeding in the above manner, that not only the recovery of the different cell bands which were layered in cavity 2, is performed at a much higher velocity than was possible with the known technique, but also happens so that the
20 bands are compressed in cavity 2 (instead of expanding, as in the known technique) and can therefore be collected separately in a clear manner, being expelled to the exterior of cavity 2 without undergoing any hydrodynamic disturbance, i.e. without cell band remixing with those immediately adjacent, thus allowing a clear and rapid individuation of cells of differing densities initially
25 present in the cell population examined.

In order to illustrate the use of the device, the separation of cellular populations from human bone marrow will be taken as an example.

It is known that human bone marrow contains various cellular populations with differing densities (see "Haematologica", January-February 1997): some cellular
30 types are light (low density), others are of intermediate density and others are heavy (high density). In order to separate said cellular populations and collect

them separately, a cellular separator is used as described above with reference to Figures 2 to 4, in which cavity 2 has a volume of 90ml, and the various ducts are 2mm in diameter.

90ml of liquid (with a continuous density gradient between 1.050 and 1.100g/l) is 5 introduced into the separator device through duct 4. Then 5ml of bone marrow monodispersed cellular suspension is introduced above the liquid already present through channel 8.

The cellular separator device, thus loaded, is then centrifuged at 400 g for 1 hour, to obtain successive stratification of the different cellular populations in discrete 10 bands of the suspension present inside cavity 2 of the device. For the separate collection of the various cellular fractions, so separated, a heavy water immiscible liquid is introduced at the base through duct 4 at a flow velocity of 2ml/min, while simultaneously pumping air from the top, through tube 8, at the same rate (2ml/min). Thus the cellular fraction of intermediate density, present at the height 15 of the duct 9 exit holes located halfway in cavity 2, is compressed and forced out through these and is collected in a few minutes (approx. 5 minutes), without remixing with the other cellular fractions due to hydrodynamic disturbances.

Similarly, to collect the light cellular fractions situated in the upper part of the suspension, or the heavy ones placed in the lower part of the same suspension, it 20 is sufficient to move the strata of differing densities towards the top or the bottom (using the peristaltic pump connected to ducts 4 and tube 8) so as to obtain the cellular fraction desired, at the height of the lateral exit holes (ducts 9) after which, in cavity 2, heavy liquid is introduced from the base (using the peristaltic pump) and air from the top to collect the desired cellular fraction through the lateral exit 25 holes mentioned.

CLAIMS

1. A device for the separation of human or animal cells of different densities, from cellular dispersions in which they are contained, having a base and a top and containing an elongated chamber whose cross section decreases from the base towards the top of the device which contains at least a first channel, one end of which opens on the inside of the said chamber near the said base and the other end connectable to a pressurised liquid source and a second channel whose end opens in the same chamber at level corresponding to the device top, characterised by the fact that there is at least an additional channel in the said device, one end of which opens at an intermediate level of the said chamber length, and the other end opens outwards from the device and in that near said base a flow deflector is provided to disperse evenly through the entire cross section of the inside chamber of the incoming fluid arriving from said first channel.
2. A separation method of human or animal cells with different densities from cellular dispersions which contain them, in which, first of all, a dispersion of a cell population is introduced into the chamber of a separator device previously filled with a liquid whose density varies from a maximum, near the device base, to a minimum near the top, and the device with cellular dispersion is subjected to centrifugation for the distribution of the cells into distinct fractions with differing densities, the method being characterised by the fact that the recovery of the separated fractions takes place by introducing a very dense water immiscible liquid at the base of the separator device while simultaneously pumping a gas, at the same pressure as said very dense liquid, to the device top so that the layered cellular fractions inside the chamber are compressed and forced out through at least one hole situated at an intermediate level of said chamber length.

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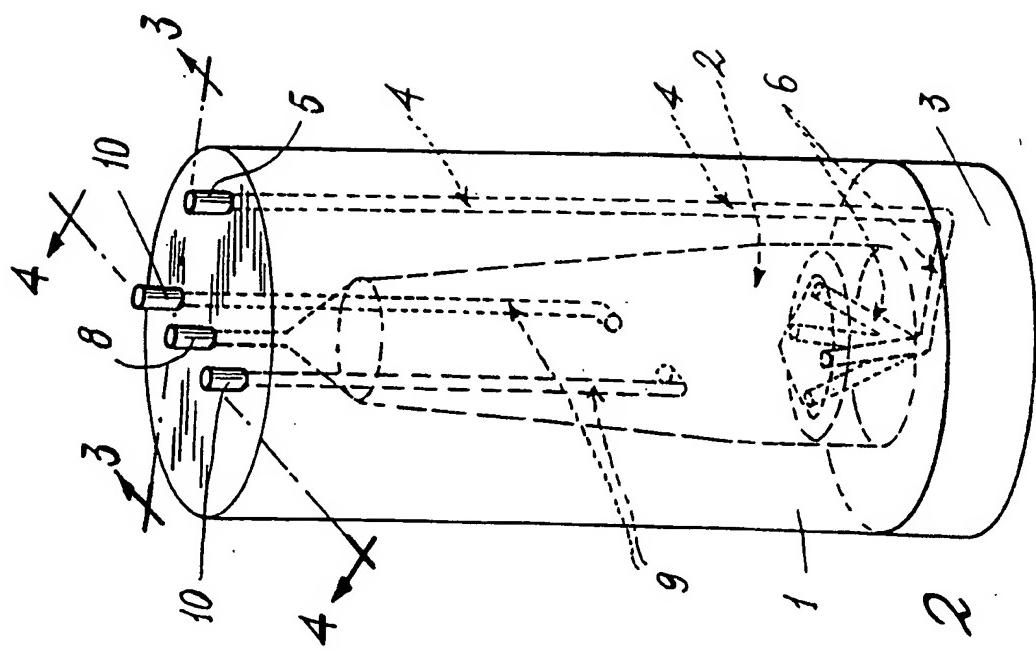
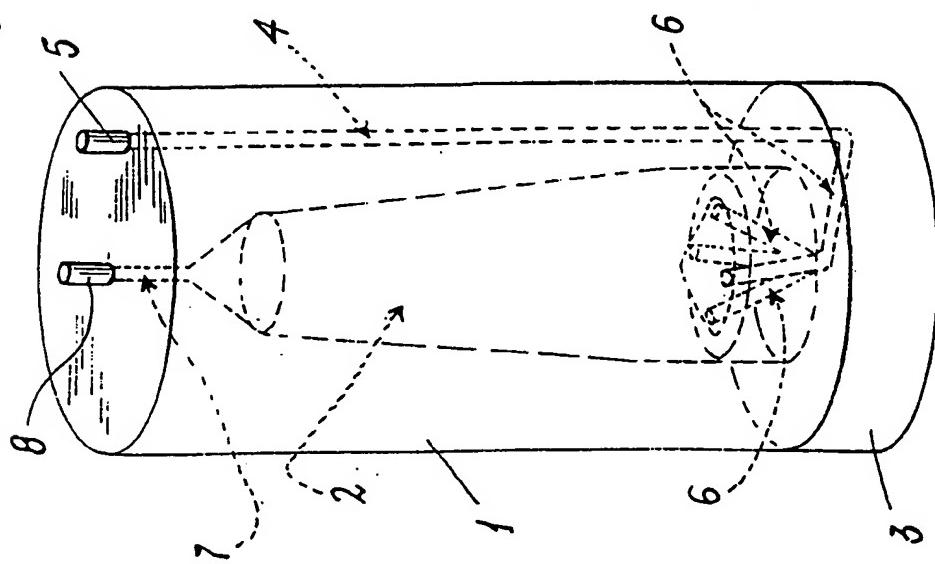


Fig. 2

Fig. 1



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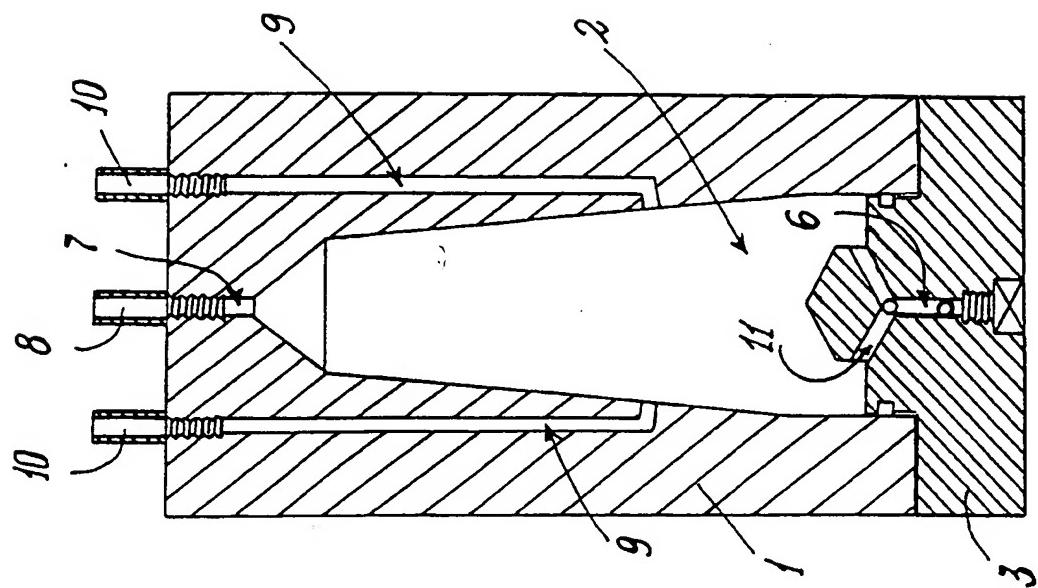


Fig. 4

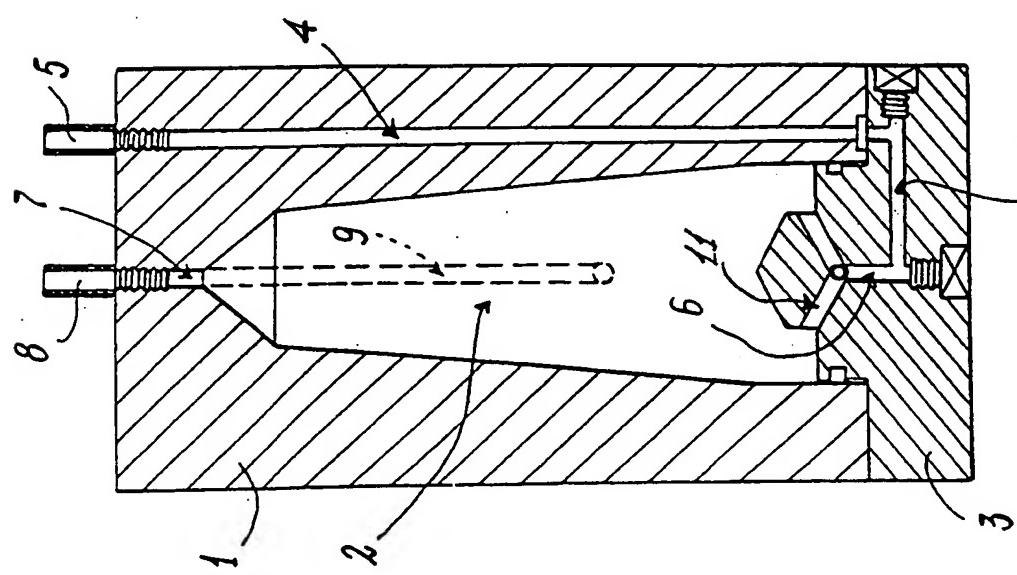


Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/EP 98/06865

A. CLASSIFICATION OF SUBJECT MATTER

G 01 N 15/00, B 04 B 5/04, B 04 B 7/02, G 01 N 33/48

According to International Patent Classification (IPC) or to both national classification and IPC 6

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G 01 N, B 04 B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5100372 A (HEADLEY, T.D.) 31 May 1992 (31.05.92), abstract, column 2, lines 47-62, fig. 1. --	1, 2
A	US 4146172 A (CULLIS, H.M.) 27 March 1979 (27.03.79), abstract, column 2, lines 40- 55, claims, fig. 1-11. --	1, 2
A	EP 0486480 A2 (BAXTER INT. INC.) 20 May 1992 (20.05.92), abstract, fig. 1, 2. --	1, 2
A	EP 0475457 A1 (TEIJIN LIMITED) 18 March	1, 2



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search
25 February 1999

Date of mailing of the international search report

12.03.99

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INTERNATIONAL SEARCH REPORT

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International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	1992 (18.03.92), abstract, page 3, line 25 - page 5, line 57, claims, fig. 1,2. --	
A	EP 0420153 A1 (TEIJIN LIMITED) 03 April 1991 (03.04.91), abstract, claims, fig. 2,3,4. --	1,2
A	EP 0198462 A2 (ASSAF PHARM. IND. LTD.) 22 October 1986 (22.10.86), abstract, page 3, line 2 - page 6, line 22; fig. 1-6. --	1,2
A	WO 96/07097 A1 (ACTIVATED CELL THERAPY, INC.) 07 March 1996 (07.03.96), abstract, claim 1, fig. 1,6. --	1,2
A	SITAR, G. et al. Physical procedures for the separa- tion of blood and marrow cells. Haematologica, Jan.- Feb. 1989, Vol. 74, pages 95-111. --	1,2
A	TULP, A. A separation chamber to sort cells, nuclei, and chromosomes at moderate g forces. II. Studies on velocity sedimentation and equilibrium density centri- fugation of mammalian cells. An International Journal of Analytical and Preparative Methods. 1981, Vol. 117, pages 354-365. -----	1,2

ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

ANNEXE

au rapport de recherche international relatif à la demande de brevet international n°

PCT/EP 98/06865 SAE 215870

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Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
US A 5100372	31-03-92	Keine - none - rien	
US A 4146172	27-03-79	CA A1 109749 DE A1 2845364 DE D1 2845364 DEU D1 2845364 FERRAI A1 2406478 GBR D1 2406478 GBR DD 20060388 GBR E 20060588 GBR GB 20060588 JP A2 04064893	02-12-80 19-04-79 07-09-89 08-04-79 18-05-79 11-10-84 04-02-80 04-02-80 04-02-80 04-02-80
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